REMARKS

Claims 1-22 and 40-47 are pending. Claims 6-15, 18, 19, and 40-47 are withdrawn following a restriction requirement. Claims 1-5, 16, 17, and 20-22 are rejected under 35 U.S.C. § 112, first paragraph. Claims 4, 16, and 22 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite. Claims 1, 16, 17, and 20-22 are rejected under 35 U.S.C. § 102(b) as being anticipated by Marshall et al., U.S. Patent No. 4,933,365 ("Marshall"). Claims 1-5 and 16 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Madsen et al., *Journal of Investigative Dermatology* 99:299-305 (1992) ("Madsen"), in view of Spurway et al., *FEBS Letters* 404:111-114 (1997) ("Spurway"). Claims 17 and 20-22 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Madsen in view of Spurway and further in view of Marshall.

Claim Amendments

Claims 3-16, 18-19, and 23-47 are canceled. Applicants reserve the right to pursue the canceled subject matter in one or more continuation or divisional applications. Claim 1 has been amended to incorporate limitations of previously presented claims 5 and 16. No new matter is added by these amendments.

Objection to the Specification

The Office objects to the specification because the Brief Description of the Drawings is after the Detailed Description of the specification. In response to this objection, Applicants have rearranged the specification. No new matter is added by this amendment. This objection may be withdrawn.

Objection to the Claims

The Office objects to claim 3 because the name of the chemical compound "carnitine" is misspelled. This claim has been canceled, and the objection is therefore moot.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1-5, 16, 17, and 20-22 are rejected under 35 U.S.C. § 112, first paragraph. The Office asserts that, while the specification is enabling for the treatment of a chronic and/or atopic skin disease, it does not reasonably provide enablement for the prevention of such a disease. Applicants have amended claim 1 to specify a method of treatment, and this ground for rejection may therefore be withdrawn.

Further, claim 4 has been rejected as lacking a written description. This claim has been cancelled, and the rejection is therefore moot.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 4 and 16 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. These claims have been canceled, and this portion of the rejection is moot.

Claim 22 is also rejected as indefinite due to the recitation of "preferably" and the trade name Vaseline. Applicants have deleted both "preferably" and "Vaseline" from claim 22. This ground for the rejection may also be withdrawn.

Rejection under 35 U.S.C. § 102(b)

Claims 1, 16, 17, and 20-22 are rejected under 35 U.S.C. § 102(b) as being anticipated by Marshall et al., U.S. Patent No. 4,933,365 ("Marshall"). The Office asserts that Marshall teaches compounds that are inhibitors of fatty acid oxidation (col. 3, lines 32-59) and the PLA_2 enzyme (col. 7, line 50) and that, as such, they are useful in the

treatment of conditions mediated by products of the oxidation of arachidonic acid (e.g., psoriasis and related skin inflammation). This rejection is respectfully traversed.

The current claims are directed to methods for treating psoriasis by administering etomoxir. As indicated above, the Office has cited two passages in the Marshall reference as supporting the rejection. Looking first to Marshall at column 3, lines 32-59, Applicants note that this passage simply refers to a variety of compounds having hypoglycemic and hypoketonemic activity taught in U.S. Patent Nos. 4,324,796 and 4,337,267 and their use for disorders such as diabetes.

The other cited passage in Marshall (col. 7, lines 49-60) teaches that the disclosed compounds are useful in treatment of conditions mediated by oxidation products of arachidonic acid because of their activity as Phospolipase A_2 inhibitors (PLA_2). This passage states (emphasis added):

> The compounds of the invention, by virtue of their ability to inhibit activity of PLA_2 enzyme, are useful in the treatment of conditions mediated by products of the oxidation of arachidonic acid. Accordingly, the compounds are indicated in the prevention and treatment of such conditions as allergic rhinitis, allergic bronchial asthma and other naso-bronchial obstructive air-passageway conditions, other immediate hypersensitivity reactions, such as allergic conjunctivitis; and various inflammatory conditions such as those present in rheumatoid arthritis, osteoarthritis, tendinitis, bursitis, psoriasis (and related skin inflammation) and the like.

Marshall therefore teaches that PLA_2 inhibition is necessary to treat conditions that would include psoriasis. The present claims, however, are not directed to treatment using a PLA_2 inhibitor. Rather, claim 1 is directed to treatment with etomoxir. As indicated in the present specification, this compound is an inhibitor of carnitine-palmitoyl-transferase-1 (CPT-1). Further, as attested to in the attached Declaration of Dr. John Nieland, etomoxir does not possess any significant PLA_2 inhibitory activity. Marshall does not teach that CPT-1 inhibitory compounds are useful in the treatment or prevention of psoriasis.

Applicants further note that the synthesis of etomoxir is described in Example 11 of Marshall but that PLA_2 inhibition by this compound is not described (see, for example, Table 1, Example 12, col. 20, lines 27-38). Nor is this compound shown to provide any characteristics indicative of therapeutic effect (see, for example, Tables 2-8). Because etomoxir is not a PLA_2 inhibitor, one reading Marshall would not consider etomoxir to be a useful treatment for psoriasis or any of the other disclosed conditions.

The rejection under 35 U.S.C. § 102(b) may be withdrawn.

Rejections under 35 U.S.C. § 103(a)

Claims 1-5 and 16 are further rejected under 35 U.S.C. § 103(a) as being unpatentable over Madsen (*Journal of Investigative Dermatology* 99:299-305 (1992)) in view of Spurway (*FEBS Letters* 404:111-114 (1997)). The Office asserts that Madsen teaches that altered transport and/or metabolism of fatty acids are involved in psoriasis and Spurway teaches that etomoxir is an inhibitor of mitochondrial long chain fatty acid oxidation. This rejection is respectfully traversed.

To support this rejection, the Office cites page 304, col. 2, paragraph 1 of Madsen. This paragraph reads (emphasis added):

> At present, there is mounting evidence suggesting that fatty acid binding proteins present in the skin play a role in the storage and transport of fatty acids [19, 37, 38], as this tissue lacks some enzymes of the essential fatty acid metabolism. As a result, the skin must import γ-linoleic acid, dihomo-γ-linoleic acid, and arachidonic acid—produced primarily in the liver—from the blood. The dramatic up regulation of PA-FABP observed in psoriatic keratinocytes points to an altered transport and/or metabolism of fatty acids in this disease, an observation that is line with several reports showing that the skin of psoriatic patients contains increased total lipids, phospholipids as well as free arachidonic acid, and derived products such as leukotriene B4 [20-22]. Further biochemical studies are needed to assess the precise function of PA-FABP in fatty acid metabolism.

At best, therefore, Madsen teaches that the upregulation of a protein termed PA-FABP might correlate with altered transport and/or metabolism of fatty acids, because PA-FABP is upregulated in psoriatic keratinocytes relative to primary normal keratinocytes. To conclude that this is true, however, would be in error, as other references in this field published subsequent to Madsen contradict this conclusion.

Applicants provide herewith, for example, Arechalde et al., *BioDrugs* 13(5): 327-333 (2000) ("Arechalde"; Exhibit 1). This reference, published after Madsen, shows that retinoic acid ("RA") is useful as a treatment for psoriasis, but a study by Larsen et al., *Exp. Dermatol.* 3(5): 212-218 (1994) ("Larsen"; Exhibit 2), also provided herewith and published after Madsen, shows that the topical application of RA leads to the rapid induction of PA-FABP transcripts in patients. A reading of Madsen, of course, would suggest that a psoriasis treatment should decrease PA-FABP synthesis. Thus, Arechalde and Larsen show that the actual effect of a psoriasis treatment on PA-FABP levels is wholly contradictory to what would be predicted by the teaching of Madsen. It is clear therefore that Madsen's hypothesis regarding blocking of fatty acid metabolism to treat psoriasis was speculative and inconsistent with other teachings in this area.

Further, the Office correctly notes that Madsen does not teach that CPT-1 inhibitors, such as etomoxir, would be useful in treating psoriasis and thus fails to provide guidance as to other pathways by which the transport and/or metabolism of fatty acids might be regulated. Madsen is therefore lacking in a number of important respects, none of which is provided by the secondary reference by Spurway.

Spurway discloses the effects of etomoxir only in hepatocytes and adipocytes, cell types not directly relevant to psoriasis. Moreover, Spurway teaches that the effects of etomoxir vary in the two cell types studied and further that, at higher concentrations in hepatocytes, the observed effects result from a CPT-1-independent pathway. Spurway does not teach that effects of etomoxir in hepatocytes and adipocytes could or should be extrapolated to skin cells (e.g., the keratinocytes studied in Madsen), and Spurway's

results predict different effects of etomoxir in different cells. Spurway therefore does not forecast what, it any, effect etomoxir would have in, for example, keratinocytes or any other cell type relevant to psoriasis.

Further, the cell type-specific nature of etomoxir is consistent with the biology of the CPT-1 enzyme that this compound inhibits. It is known in the art that short sequences at the N-terminus of CPT-1 can vary in different isoforms (e.g., liver and muscle isoforms) and that these variations can influence the kinetic characteristics of the proteins. As evidence of this fact, Applicants provide herewith a reference by Zammit et al., *Biochemical Society Transactions* 29 (2), 287-292 (2001) ("Zammit"; Exhibit 3). Zammit, as an example, teaches that native liver CPT-1 and native muscle CPT-1 have different K_m values for the binding of carnitine and that mutation of residues 3-18 of each isoform results in divergent effects (see Figure 3, page 290). Zammit therefore teaches that CPT-1 isoforms from different tissues have different kinetic profiles (e.g., substrate binding). As a result, Spurway, whose teaching is limited to the effects of etomoxir in hepatocytes and adipocytes, cannot be used to teach that etomoxir would have the desired effect in psoriasis-related cells, such as keratinocytes.

In sum, Madsen and Spurway, even in combination, do not suggest the claimed invention, and this ground for the § 103 rejection should be withdrawn.

Claims 17 and 20-22 also stand rejected, under 35 U.S.C. § 103(a), as being unpatentable over Madsen in view of Spurway and further in view of Marshall. This rejection is also respectfully traversed.

As indicated above, Madsen and Spurway do not form the basis for a *prima facie* case of obviousness. And Marshall cannot be used to remedy the deficiencies of Madsen and Spurway. The Office cites Marshall as teaching topical administration of the disclosed compounds. But Marshall, in fact, teaches a variety of modes of administration for a variety of different compounds. Marshall provides several different compounds represented by two different general formulas for the treatment of several different

diseases, as well as a teaching of essentially all traditional modes of compound administration, including oral, parenteral, inhalation, insufflation, topical, and intravenous approaches.

Moreover, in the Examples, Marshall discloses several different formulations for the application of the compounds to a patient. Only one of them concerns a topical formulation. Further, the topical applications exemplified for compounds other than etomoxir can also not be regarded as preferred applications because Examples 16 and 17 disclose an oral application of the drugs and Example 19 an intravenous administration of the drug. Marshall therefore does not call out topical administration as a preferred route of administration, and certainly does not suggest that etomoxir should be formulated for topical administration for treatment of psoriasis.

This ground for the § 103 rejection should also be withdrawn.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested. Enclosed is a Petition to extend the period for replying to the Office action for three months, to and including October 11, 2008 and a check in payment of the required extension fee.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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Management of Psoriasis
The Position of Retinoid Drugs

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Contents

Abstract

Oral synthetic retinoids have been established as effective systemic therapy for psoriasis since their introduction for clinical use in the 1970s; a compound for topical use, tazarotene has been recently marketed. Despite the demonstrated clinical success of retinoid therapy in psoriasis, its mechanism of action has not been fully elucidated, and investigators are confronted with two paradoxes. One is that the binding of retinoids to nuclear retinoic acid receptors (RARs) does not match their therapeutic efficacy: acitretin activates the three receptor subtypes, RAR-α, -β and -γ, without measurable receptor binding, whereas tazarotene preferentially binds to and activates RAR-β and -γ in preference to RAR-α. The other is that there is already increased formation of retinoic acid in the psoriatic lesion. Answering these questions should result in better use of these drugs in the treatment of psoriasis.

Oral administration of acitretin remains one of the first therapeutic choices for severe psoriasis, particularly in association with ultraviolet light therapy, of which it may decrease the carcinogenic risk. Topical tazarotene is suitable for moderate plaque psoriasis. Its efficacy and tolerability can be enhanced by the addition of topical corticosteroids; its irritative potential is counterbalanced by a sustained therapeutic effect after the treatment is stopped.

1. Retinoids for Psoriasis: Why?

Retinoids used for the management of psoriasis are synthetic analogues of retinoic acid, the natural ligand of nuclear retinoic acid receptors (RARs). Despite the demonstrated clinical efficacy of retinoid therapy in psoriasis, its mechanism of action has not been fully established (reviewed by Saurat[1]). Retinoids are said to exert their therapeutic effect by modulating 3 major pathogenic features of psoriasis: disturbed keratinocyte differentiation, keratinocyte hyperproliferation and tissue infiltration by inflammatory cells. The primary molecular target for these effects is not established. According to current concepts of the pathogenesis of psoriasis, T lymphocytes are thought to trigger the chain of cellular and molecular events that induce the psoriasis phenotype; epidermal keratinocytes participate in establishing the appropriate cytokine milieu.[2] It has been suggested that epidermal hyperplasia is driven by T lymphocytes and, parallel to hyperproliferation, resistance of keratinocytes to apoptosis also accounts for this hyperplasia.[2] The mechanism by which retinoids interfere with these events is not established, and is thus hypothetical. Retinoids may alter the cytokine production profile by T lymphocytes, interfere with keratinocyte responsiveness to cytokines, or abolish the resistance of keratinocytes to apoptosis. Answering these questions and identifying the molecular pathways involved should result in better use of these drugs in the treatment of psoriasis.

The discovery of nuclear RARs was an important advance which may lead to a unifying theory of retinoid action and provide the means to design retinoids with specific properties for targeted use. So far, the field is confronted by 2 paradoxes. One is that acitretin, the synthetic retinoid used for oral treatment of psoriasis, activates all 3 nuclear retinoic acid receptor subtypes (RAR-α, -β and -γ) without measurable receptor binding (reviewed by Saurat[1]). The other is that there is already increased formation of retinoic acid in the psoriatic lesion,[3] which may be triggered by interferon-γ;[1] therefore, additional loading with exogenous retinoids appears therapeutically unsound.

2. Retinoids for Psoriasis: Which?

Until recently, treatment of psoriasis with retinoids has been restricted to systemic administration; however, a topical drug is now available.

Three synthetic retinoids have been marketed for oral use: the aromatic retinoid ester etretinate, the free-acid derivative of etretinate, acitretin, and 13-cis-retinoic acid, isotretinoin. Etretinate and its active metabolite, acitretin, have an established role in the treatment of psoriasis; etretinate was first reported to be effective in psoriasis in 1975 and acitretin in 1984. Isotretinoin has a less marked antipsoriatic effect.[4,5]

Tazarotene is the first topical retinoid demonstrated to be both effective and tolerable in treating plaque-type psoriasis.[6] This interesting development has renewed interest in the topical retinoid treatment of psoriasis, after previous failures to treat the disease with topical retinoic acid.

The choice between topical and oral treatment is based on the severity and extent of the disease.

3. Retinoids for Psoriasis: How?

3.1 Etretinate and Acitretin

3.1.1 Pharmacokinetics and Their Implications

Etretinate is an ethylester that undergoes extensive hydrolysis in the body to yield the corresponding acid metabolite. It appears from cellular and molecular pharmacology studies that the pharmacologically active compound of etretinate is acitretin, and therefore etretinate may be considered as a prodrug.[7] In animal models[7] and in clinical studies in patients with severe keratinising disorders,[8] acitretin is equally effective compared with etretinate. However, acitretin has a profound pharmacokinetic advantage because it is eliminated more rapidly than etretinate.[9] Etretinate is approximately 50 times more lipophilic than acitretin and binds strongly to plasma proteins, particularly lipoproteins and albumin. This fact has a profound influence on the respective pharmacokinetic properties of the 2 drugs.[9]

When etretinate or acitretin is taken with a meal, the absorption of the drug is increased 2- to 5-fold

and the amount absorbed is more consistent.[9,10] It is therefore recommended that etretinate or acitretin be taken with the main meal of the day. However, even when the drugs are taken with food, they are absorbed to a variable extent. This leads to marked interindividual variations in plasma retinoid concentrations, which appears not to depend upon bodyweight.[4]

The main adverse effect associated with synthetic retinoids is teratogenicity, and the length of time that these drugs are present in the body is thus of great interest. Etretinate is stored in adipose tissue, including subcutaneous fat, from which it is released slowly. It thus has a long terminal elimination half-life of up to 120 days. In contrast, under identical conditions, acitretin carries a negatively charged group and, being so much less lipophilic than etretinate, does not accumulate in adipose tissue and is therefore eliminated from the body more rapidly. Acitretin has an elimination half-life of 2 days.[11]

Initial publication of data indicating that etretinate can be formed in patients receiving acitretin[12] has been confirmed by several similar reports.[11,13] This esterification seems to occur when acitretin is taken simultaneously with alcohol.[13] This finding prompted the manufacturer to extend the time of compulsory contraception in patients taking acitretin to 2 years, as it is for etretinate.[5] Indeed, the US Food and Drug Administration (FDA) recently approved the therapy contraceptive period of at least 3 years for acitretin, based on the pharmacokinetics of acitretin and etretinate in clinical trials and on previous safety experience with etretinate. The pharmacokinetic advantages of acitretin over etretinate still hold true, however, for all women who strictly avoid alcohol during treatment and for 2 months thereafter.[11]

For all these reasons acitretin represents a therapeutic alternative with greater clinical potential than etretinate; in June 1997, acitretin was approved by the FDA as a substitute for etretinate for similar indications.[11]

3.1.2 Therapeutic Profile

Several randomised multicentre trials have been performed to ascertain the efficacy of acitretin on plaque-type[14-18] and pustular psoriasis and to compare its efficacy with that of etretinate.[14] Acitretin appears to be as effective as etretinate[8] and can be used in the same combination regimens.[19] Adverse effects appear to be similar in quality and incidence.

The best results have been obtained in palmoplantar or generalised (Von Zumbusch) type pustular psoriasis in which etretinate and acitretin are considered to be the treatments of first choice.[20,21] Both localised and generalised pustular psoriatic lesions as well as those of erythrodermic psoriasis are cleared more frequently with etretinate or acitretin monotherapy than with most other therapies.[20-22]

Rebound does not usually occur after stopping acitretin or etretinate treatment,[23] and reintroduction of the drug when it does occur produces a beneficial response.[22]

The most common form of psoriasis, plaque-type psoriasis, responds variably to both drugs. Although complete clearing of the lesions is achieved in only about 30% of treated patients, a significant improvement is obtained in a further 50%.[24-28] The decrease in the Psoriasis Area and Severity Index (PASI) score is approximately 60 to 70%, depending on the dosage.[8] Approximately 20% of patients may be considered treatment failures.[8,27]

One of the main reasons for treatment interruption is initial worsening of the disease with an increase in erythema. Another is an increase in the extent of the lesions, which may occur within a few days of the start of treatment at dosage of 0.5 to 1.0 mg/kg/day, i.e. 30 to 70 mg/day. A therapeutic dosage scheme using initially low dosages (10 mg/day) of etretinate[29] or acitretin,[30] followed by progressively increasing the dosage, seems to avoid this initial worsening.[4]

In order to define the efficacy of acitretin as monotherapy, different dosages (10 to 75 mg/day) were compared in a double-blind fashion with placebo in patients with psoriasis. At the end of an 8-week treatment period, acitretin at dosages of 25

and 50 mg/day[31] and 50 and 75 mg/day[32] was superior to placebo in two studies. The efficacy of 10 mg/day was not significantly different from that of placebo in either study.[4]

Total clearing of the lesions usually requires a combination of treatments such as retinoids plus topical corticosteroids,[33] dithranol (anthralin)[34] or photochemotherapy (psoralen plus ultraviolet A; PUVA).[35-37]

3.1.3 Combination with Ultraviolet Therapy

One of the major developments for the treatment of widespread plaque-type psoriasis in recent years has been the combined use of PUVA photochemotherapy with the retinoids etretinate or acitretin (Re-PUVA therapy). The effects of etretinate and PUVA appear to be additive.[35,36,38]

More recently, studies using acitretin clearly demonstrated the superiority of Re-PUVA. In a double-blind comparative trial,[19] patients with psoriasis received either retinoid (acitretin or etretinate 50 mg/day) or placebo without PUVA for 2 weeks. This monotherapy phase was followed by a combined therapy phase as follows: retinoid (acitretin or etretinate 25 mg/day) or placebo, plus PUVA given 3 times a week until remission. Although etretinate plus PUVA was better than placebo plus PUVA, statistically significant differences in the number of PUVA exposures and total dose of ultraviolet A (UVA) until remission were shown only between acitretin plus PUVA and placebo plus PUVA. The efficacy of acitretin was confirmed in another study which showed that the mean cumulative UVA dose was 42% less in the acitretin plus PUVA group than in the placebo plus PUVA group.[39]

The main advantages of the Re-PUVA combination are the acceleration of the response rate of psoriatic lesions and the clearing of lesions in patients that could not be cleared with the retinoid or PUVA alone. A further advantage is that the radiation exposure necessary to produce remission is significantly lower with Re-PUVA. The lower radiation dosage is likely to diminish the risk of PUVA-induced carcinogenic effects, which are clearly UVA-dose dependent.[40] Oral retinoids can also be combined with bath PUVA (psoralen bath plus UVA), which also allows a reduction in the effective dose of UVA.[41]

The combination of etretinate[42] or acitretin[43,44] with ultraviolet B (UVB) phototherapy has been shown to be more effective in patients with psoriasis than retinoid or UVB phototherapy alone.

3.1.4 Treatment of Patients with HIV Infection

The frequency of psoriasis in patients with HIV infection does not differ significantly from the occurrence of the disorder in patients who do not have HIV infection. However, the psoriasis in HIV-infected patients tends to be more aggressive, and treatment poses a unique challenge. Topical agents are not usually sufficient to control the disease process in this group of patients. Potentially immunosuppressive treatments for psoriasis, including phototherapy and the use of corticosteroids, methotrexate and cyclosporin, are contraindicated. Eleven patients with psoriasis associated with HIV infection were enrolled in a 20-week treatment protocol. Six (54%) of the 11 achieved good to excellent responses using acitretin monotherapy. Parameters of immunosuppression were not exacerbated by acitretin therapy. Both skin and joint manifestations responded to acitretin therapy in most patients. Optimal results were achieved with a dosage of 75 mg/day. The adverse effects were moderate and well tolerated. Thus, acitretin does not appear to have immunosuppressive properties. In fact, its mode of action in psoriasis is unknown, regardless of HIV status.[45]

3.1.5 Adverse Effects and Contraindications

There is one absolute contraindication to use of retinoids: pregnancy. Retinoids are potent teratogens,[46] and women must avoid conception not only during treatment but also for a certain period after stopping therapy. Calculation of the recommended post-therapy contraception period can be based on the elimination half-life of the retinoid; i.e. it takes 7 times the half-life to eliminate 99% of the drug from the body. The teratogenic potential of plasma and tissue concentrations of the remaining drug is assumed to be negligible. However, this approach seems not to be universally accepted, which explains

the differences in the pregnancy warnings between countries.

In most countries, however, the post-therapy contraception period is 2 years for etretinate. A 2-month period was initially proposed for acitretin but, since it is now apparent that acitretin may metabolise to etretinate, it is logical to extend the contraception period for acitretin to that for etretinate. When acitretin is given to women of childbearing age, consumption of alcohol should be forbidden because alcohol appears to be a major factor in the formation of etretinate from acitretin (see section 3.1.1).

Other adverse effects of retinoids are generally preventable or manageable through proper patient selection, dosage adjustments and routine management. Mucocutaneous adverse effects such as cheilitis and hair loss are the most common dose-dependent adverse effects; these require dose reduction in some patients. Hepatotoxicity, serum lipid alterations, pancreatitis and possible skeletal effects are less common adverse effects. Careful monitoring is necessary in patients with a history of hepatitis, diabetes mellitus, hyperlipidaemia, atherosclerosis or pancreatitis. Acitretin or etretinate should not be given to patients with serious retinal disorders.[4,47]

3.2 Isotretinoin

Isotretinoin has a lesser effect on psoriasis than acetretin or etretinate, although some efficacy has been shown in combination with PUVA.[5,48] Nevertheless, some dermatologists continue to use isotretinoin in women with psoriasis who need systemic retinoids, to avoid the long post-acitretin contraception period.[49]

Five patients with generalised pustular psoriasis treated with oral isotretinoin showed significant improvement in a small study.[50] In an open study, 11 patients with generalised pustular psoriasis were treated with oral isotretinoin 1.5 to 2 mg/kg/day; pustular lesions were completely cleared in 10 of the 11 patients. The systemic symptoms completely resolved during this time. Follow-up of these patients for up to 2 months showed that the majority subsequently required an alternative agent to achieve satisfactory control of their psoriasis. It appears that isotretinoin aborted the attack of generalised pustulation but was not effective in treating the residual psoriasis when used alone.[51]

In a nonblinded study comparing isotretinoin with etretinate, 10 patients who had psoriasis affecting 20 to 50% of their body surface area were treated with isotretinoin 1.5 mg/kg/day for at least 8 weeks, and 19 patients who had psoriasis affecting 40 to 90% of their body surface were treated with etretinate 0.75 mg/kg/day for at least 8 weeks. 18 of 19 patients treated with etretinate had either a complete or a moderate response, while only 4 of 10 patients treated with isotretinoin were moderate or complete responders. Thus, analysis showed a significant difference in favour of the etretinate-treated group.[51]

Because of teratogenicity concerns, women of child-bearing potential must have a negative pregnancy test and practise effective contraception during and for 1 month after completing therapy with isotretinoin. The mandatory 1-month post-therapy contraceptive period is necessary in order to afford a sufficient safety margin, since plasma concentrations of isotretinoin return to physiological levels within 10 days of therapy completion.[52]

3.3 Topical Tazarotene

Tazarotene is a synthetic analogue and the first topical retinoid that has been demonstrated to be both effective and tolerable in treating plaque-type psoriasis. Tazarotene gel is available in 2 concentrations, 0.05 and 0.1%.

The molecular structure of tazarotene was engineered in such a way as to make it selective for the RAR class of nuclear receptors over retinoid X receptors (RXR), with preferential activation of RAR-β and RAR-γ over RAR-α.[6] There are, however, no data indicating that this receptor binding profile is involved in the antipsoriatic activity of tazarotene.

On the basis of current available data, tazarotene can be used for the treatment of chronic, stable, plaque-type psoriasis on the trunk or limbs, covering up to 20% of the body surface area.[53]

The clinical efficacy and tolerability of topical tazarotene have been investigated in vehicle-controlled and active-controlled studies. It has been demonstrated to be significantly more effective than vehicle, and comparable in efficacy to fluocinonide 0.05% but with a more sustained therapeutic effect after treatment is stopped.[54] In clinical trials, patients generally experienced a clinical response within 4 weeks of starting tazarotene treatment, and improvement was maintained for up to 12 weeks after stopping therapy.[54]

Ongoing studies are evaluating the use of tazarotene in combination with other antipsoriatic medications. The efficacy and tolerability of tazarotene can be enhanced by the addition of topical corticosteroids. When used in combination with broad-band UVB phototherapy, tazarotene reduces the amount of UV light required to treat plaques.[55]

Tazarotene is associated with skin irritation in many patients, which can be managed by reducing the concentration or frequency of application, or by adding a topical corticosteroid.[53]

The irritative potential of tazarotene appears to be counterbalanced by a sustained therapeutic effect after treatment is stopped. This may prove to be a major advantage for the drug; however, it requires confirmation in studies aimed at appropriately defining the magnitude of this effect.

4. Conclusions

So far, the major advances in the molecular pharmacology of retinoids have not been translated into adequate therapeutic answers to the challenge of treating patients with psoriasis. The teratogenic risk has clearly shifted the interest of drug developers towards topical compounds, the first of which, tazarotene, although useful, was not a breakthrough. It is hoped that retinoids with a specific pharmacological profile, for instance those that dissociate antiproliferative activity from others, will soon be developed.

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Retinoic acid induces expression of PA-FABP (psoriasis-associated fatty acid-binding protein) gene in human skin *in vivo* but not in cultured skin cells

Grønhøj Larsen F, Voorhees JJ, Åström A. Retinoic acid induces expression of PA-FABP (psoriasis-associated fatty acid-binding protein) gene in human skin *in vivo* but not in cultured skin cells.
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Abstract: PA-FABP (psoriasis-associated fatty acid-binding protein) is a new member of a group of low-molecular-weight proteins that are highly up-regulated in psoriatic skin and that share similarity to fatty acid-binding proteins. In this study we demonstrate that PA-FABP transcripts are expressed in human skin *in vivo* and that topical application of 0.05% retinoic acid (RA) cream results in a rapid induction of PA-FABP transcripts following treatment for 16 hours and persists at increasing levels after 48 and 96 h of RA treatment. The PA-FABP mRNA response to RA was reduced by approximately 50% when patients concurrently were treated with RA and 0.025% clobetasol propionate (CLO) for 48 and 96 h, whereas treatment with CLO alone resulted in PA-FABP transcript levels not significantly different from vehicle-treated skin. When comparing the effects of a well-known irritant, sodium lauryl sulfate (SLS), to those of RA and its vehicle, 0.05% RA cream but not 2% SLS in RA vehicle caused PA-FABP mRNA induction after 16 h. SLS treatment of human skin for 96 h caused a slight increase in PA-FABP transcripts, but markedly less than that observed in response to RA treatment. Incubation of cultured human keratinocytes or skin fibroblasts with RA for up to 48 h did not significantly induce PA-FABP transcripts. Expression of PA-FABP message in keratinocytes was observed to be induced by calcium and fetal calf serum (FCS), while tetra-decanoyl phorbol acetate (TPA) caused little or no induction. Taken together, the marked inducibility of the PA-FABP gene is compatible with the possibility that this gene might be important in RA-mediated regulation of human skin growth and differentiation.

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Key words: retinoic acid – gene regulation – fibroblasts – keratinocytes

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Introduction

The fatty acid-binding proteins (FABPs) belong to a multigene family of low molecular weight cytoplasmic binding proteins (≈14–15 kDa) that bind fatty acids, and are abundantly expressed in tissues specialized in the synthesis, transport, storage and utilization of fatty acids. Their putative main physiological significance is the assurance that long-chain fatty acids and derivatives, either in transit through membranes or present in intracellular compartments, are largely complexed to proteins (1). Also, since recent data indicate the involvement of fatty acids and fatty acid derivatives (e.g., eicosanoids) in cellular growth and differentiation and signal transduction pathways, their respective binding proteins may also function in these complex processes (2, 3).

The epidermis is a specialized tissue of lipid metabolism, but cannot synthezise some of the fatty acids (i.e., linoleic acid, arachidonic acid) that are important for epidermal growth and barrier functions (4). Thus, fatty acid transport in the skin and its metabolism may be facilitated by FABPs. In

fractionated non-cultured psoriatic keratinocytes there is evidence of an altered fatty acid transport and metabolism which is in line with the dramatic up-regulation of a group of FABPs including psoriasin, calgranulin A and B, and cystatin A (5, 6). Recently, Madsen et al. (7) identified a new member of this group that is highly up-regulated at the message and protein levels in non-cultured psoriatic keratinocytes and termed it PA-FABP (psoriasis-associated fatty acid-binding protein). However, nothing is known about PA-FABP gene expression and regulation in human skin. The main purpose of the present study was to examine the regulation of PA-FABP gene expression in human skin *in vivo* as well as in cultured skin cells. Since topical retinoic acid can cause an erythematous reaction clinically similar to irritant dermatitis (8–10), an important issue is the extent to which alterations induced by RA are caused by its irritant properties or by more physiologically relevant mechanisms. Therefore, we have also examined the effects of the irritant sodium lauryl sulfate (SLS) as well as the anti-inflammatory compound clobetasol propionate (CLO) on expression of PA-FABP.

Material and methods

Polymerase chain reaction (PCR) and cDNA cloning

PCR was carried out in an automated Perkin-Elmer Cetus thermocycler using 1 μg of total RNA extracted from keratome biopsies of psoriatic skin. RNA was reverse transcribed into a complementary DNA using reverse transcriptase (2.5 U) and oligo d(T)16 as primers (2.5 μM), at 42°C for 25 min, in a total volume of 20 μl buffer (5 mM $MgCl_2$, 1×reaction buffer as supplied by the manufacturer, 1 mM dXTP, 1 mM RNase inhibitor) overlaid with 70 μl of mineral oil (Gene Amp. RNA Kit, Perkin Elmer Cetus, CT, USA). Thirty-five cycles of PCR amplification (95°C, 1 min, 50°C, 1 min, 72°C, 1 min) were performed in 100 μl using Ampli Taq DNA polymerase (2.5 U), 2.6 mM $MgCl_2$, 1×reaction buffer, and 0.15 μM (final concentration) of each primer as suggested by the manufacturer. The oligonucleotide primers were synthesized by the University of Michigan DNA core facility and based upon a previously published cDNA sequence (7). Each primer had 25 nucleotides hybridizing to its template and at its 5'-end, a restriction site and 6 additional nucleotides that facilitate complete digestion with the restriction enzymes. Eco RI and Bam HI sites were contained in the forward and reverse primers, respectively. The primers were designed so as to amplify the coding region and were as follows:

Forward: 5'-gtttccgaattcACCGCCGACGCA-GACCCCTCTCTGC-3'
Reverse: 5'-caaaccggatccGGGATGATCCTAA-TTAATCCAACAC-3'

The length of the amplified DNA was determined on a 1.5% agarose gel. The amplified 633-bp region of PA-FABP was isolated from the gel, ethanol precipitated and subcloned into plasmid pSG5 (Stratagene, La Jolla, CA, USA).

Skin biopsies

For *in vivo* treatment, 0.05% retinoic acid cream, its vehicle (Ortho Pharmaceuticals, Raritan, NJ, USA) or 0.025% clobetasol propionate (CLO) ointment (Glaxo Dermatology, NC, USA) was applied once as 3×9 cm patches to buttock skin and maintained under plastic wrap for 24, 48, or 96 h prior to biopsy. When patients concurrently were treated with RA and CLO equal amounts of 0.1% RA and 0.05% CLO (approximately 250 mg of each) were mixed and applied to the skin. Subjects were also treated for 16 or 96 h under occlusion with 2% SLS. SLS was obtained from E.I. Dupont De Nemours & Company (Wilmington, DE, USA) and compounded at 2% in RA vehicle cream. After obtaining written informed consent, keratome biopsies consisting primarily of epidermis (11) were procured from the treated areas using 1% plain lidocaine as a local anesthetic. Epidermal keratomes were immediately frozen in liquid nitrogen and stored at −70°C until use. All subjects provided written, informed consent and all procedures were performed under approval of the University of Michigan Medical Center Institutional Review Board.

Cell culture

Human keratinocytes were purchased from Clonetics and expanded to about 50% confluency in 150-mm tissue culture dishes in a low Ca^{++} (0.15 mM), serum-free keratinocyte growth medium (KGM) containing epidermal growth factor (EGF) (0.1 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), bovine pituitary extract, gentamicin (50 μg/ml), and amphotericin-B (50 ng/ml) (Clonetics, San Diego, CA, USA). The effect of glucocorticoid was examined after the medium was replaced with hydrocortisone-free KGM medium 48 h prior to treatment. Cells were treated up to 48 h with 1 μM RA or calcium (2 mM) or with FCS (10%) or TPA (20 nM).

Human dermal fibroblasts were also used in this study. They were prepared from punch biopsies of buttock skin (12) and propagated in Dulbecco's

modified Eagle's medium containing 10% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were then grown to confluency in 150-mm tissue culture dishes (Corning Glass Co., Corning, NY, USA) and treated for up to 48 h.

Cells were maintained in a humidified incubator at 37°C with 5% CO_2/95% air. When cells were treated with RA, they were protected from light during the incubation period. Experiments were conducted on cells in the third through sixth passage.

Northern analysis of mRNA

RNA was isolated from cultured human skin fibroblasts, keratinocytes or frozen keratome biopsies by guanidinium isothiocyanate lysis and ultracentrifugation as previously described (13). Briefly, RNA concentrations were determined by absorbance at 260 nm and equal quantities of total RNA were electrophoretically separated in 1% formaldehyde-agarose gels containing 0.5 μg/ml ethidium bromide. Twenty micrograms total RNA was transferred to derivatized nylon membranes (Zeta-Probe, Bio-Rad, Richmond, CA, USA). Filters were baked 2 h at 80°C *in vacuo*, then prehybridized for 2–4 h at 42°C in 50% formamide, 5×SSC (1×SSC=150 mM NaCl, 15 mM sodium citrate), 50 mM sodium phosphate, pH 7.0, 1×Denhardt's solution, 250 μg/ml yeast tRNA, 100 μg/ml sonicated herring sperm DNA, and 1% SDS. Hybridization was carried out for 18 h at 42°C in the same buffer. Blots were sequentially hybridized against ^{32}P-labeled PA-FABP and cyclophilin (14) probes prepared by random priming. Quantitation of mRNA levels was performed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA), and PA-FABP message levels were normalized to cyclophilin. Filters were washed once in 0.2×SSC, 0.1% SDS at room temperature, then twice for 15 min in 0.2×SSC, 0.1% SDS at 56°C and finally once for 15 min in 0.1×SSC, 0.1% SDS also at 56°C. An adult Multiple Tissue Northern Blot was purchased from Clontech Lab., Inc. and contained 2 μg in each lane of highly pure poly $(A)^+$RNA from 8 different tissues. Autoradiography was performed using intensifying screens at −70°C.

Statistics

For each duration of time (i.e. 16, 24, 48, and 96 h) the -fold induction was compared to unity by a one-sample t-test. All p-values are two-tailed.

Results

Expression of PA-FABP mRNA in human skin in vivo

PA-FABP transcripts were detectable in untreated skin (data not shown) as well as skin treated with vehicle (Fig. 1). As shown in Fig. 1, PA-FABP mRNA was markedly and significantly increased by RA treatment compared to vehicle-treated skin at all time points studied (24, 48, and 96 h). Induction was observed as early as 16 h after topical RA treatment (mean 2.0-fold±0.23 SEM, $p=0.006$, $n=7$) (data not shown) with increasing values following treatment for 24 h (mean 2.8-fold±0.34 SEM, $p=0.006$), 48 h (mean 8.2-fold±2.5 SEM, $p=0.04$), and 96 h (mean 17.3-fold±5.5 SEM, $p=0.03$). However, a considerable inter-individual variation in -fold induction existed both at 48 (range 3 to 18) and 96 h (range 5 to 34). At these time points the induction of PA-FABP mRNA by RA was reduced by approximately 50% when patients concurrently were treated with RA and CLO. Treatment with CLO alone resulted in PA-FABP transcript levels not significantly different from vehicle-treated skin.

The treatment sites were assessed for erythema

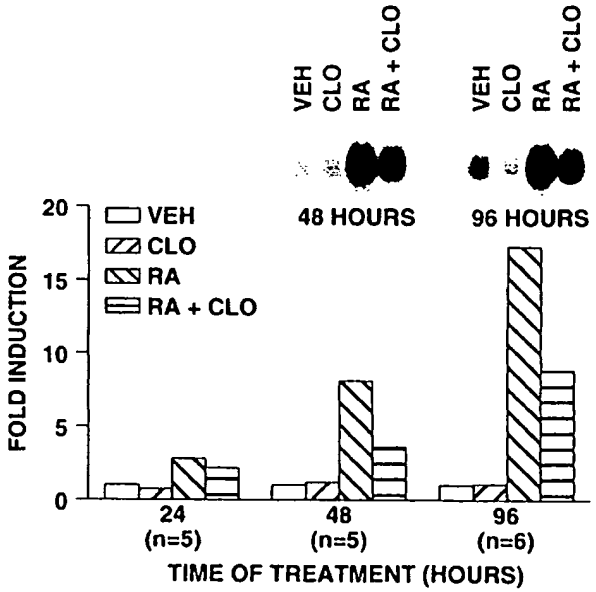

Figure 1. a) Induction of PA-FABP mRNA in human skin by topically applied retinoic acid cream (RA). Each volunteer was treated with 0.05% RA, its vehicle, 0.025% clobetasol propionate ointment (CLO), or RA and CLO in combination under occlusion for the times indicated prior to biopsy. *n* is the number of RA, CLO or RA/CLO treated patients obtained at each time point. *b)* autoradiographic bands from two representative experiments following treatment for 48 and 96 h.

using a 0-9 scale, 0 representing absent, and 9 maximal erythema (10). However, there was no correlation between degree of erythema and -fold induction by RA.

Effect of sodium lauryl sulfate on PA-FABP expression in human skin in vivo

The above data clearly demonstrate that PA-FABP expression is increased in skin treated with topical RA. To determine whether this response was specific to RA, biopsies were obtained from subjects treated with the irritant sodium lauryl sulfate (SLS) prepared in retinoic acid vehicle for 16 or 96 h under occlusion. Both 2% SDS and 0.05% RA produced a marked increase in erythema relative to vehicle after 96 h, which was comparable between the two agents. Little or no erythema was observed in response to vehicle after 96 h, nor to any of the treatments after 16 h. In 7 patients treated with SLS or RA cream for 16 h, PA-FABP transcripts were not significantly induced compared to vehicle following SLS treatment (mean 0.96-fold±0.06, p=0.54), while as mentioned above RA treatment resulted in a significant induction. Also, the induction of PA-FABP mRNA by SLS after treatment for 96 h was markedly less than that obtained in response to RA (Fig. 2).

Expression of PA-FABP in human keratinocytes and fibroblasts

Because RA induced PA-FABP mRNA *in vivo*, it was of interest to determine whether an induction also occurred in cultured human keratinocytes. Subconfluent cultures of keratinocytes were harvested, RNA extracted, and PA-FABP mRNA

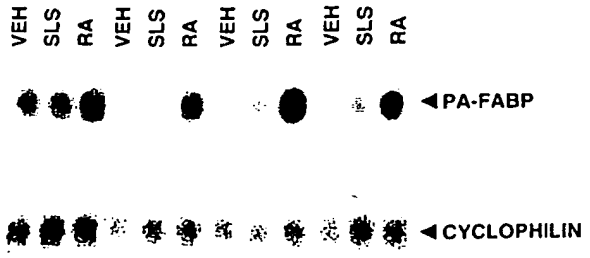

Figure 2. Induction of PA-FABP mRNA in human skin by topically applied sodium lauryl sulfate (SLS) or retinoic acid cream (RA). Four individuals were treated for 96 h under occlusion with vehicle (V), 2% SLS, or 0.05% RA. Total RNA (20 μg per lane) was successively filter hybridized with ^{32}P-labeled cDNA probes for PA-FABP and cyclophilin.

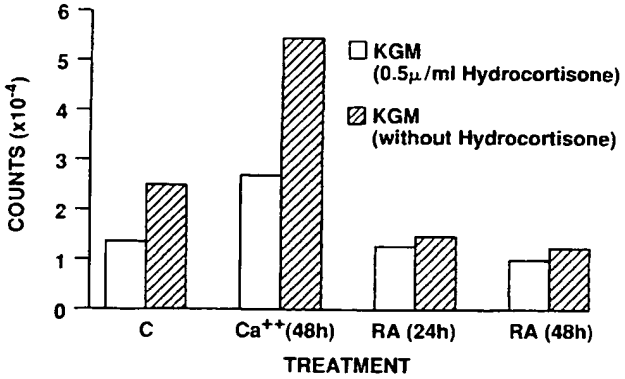

Figure 3. Induction of PA-FABP mRNA by calcium (Ca^{++}) (2 mM) and retinoic acid (RA) (1 μM) in normal adult human keratinocytes. When cells were 50% confluent, they were either maintained in hydrocortisone-containing KGM (Clonetics) growth media or the media were replaced with hydrocortisone-free KGM media 48 h prior to Ca^{++} or RA treatment.

levels were determined. In untreated keratinocytes, PA-FABP mRNA was at a low but detectable level. Treatment of keratinocytes by RA for 24 or 48 h did not induce PA-FABP mRNA when cells were grown in the presence or absence of hydrocortisone (Fig. 3). When grown in the presence of hydrocortisone, markedly lower transcript levels were observed in both unstimulated (control) and stimulated cells. Treatment with calcium for 48 h resulted in an approximately 2-fold induction of PA-FABP transcripts. In a separate experiment, incubation of keratinocytes with FCS (10%) for 48 h also induced PA-FABP gene expression (≈3-fold), while TPA (20 nM) had little or no inducible effect (data not shown). Induction of PA-FABP expression by RA was also determined in fibroblasts. In these cells, transcripts were undetectable both in untreated cells and in cells after treatment with RA (1 μM) for 24 and 48 h (data not shown).

Presence of PA-FABP mRNA in adult human tissues

To determine the tissue distribution of PA-FABP gene expression, poly(A)$^+$RNA from various adult human tissues was analyzed (Fig. 4). Of the tissues examined, heart and placenta had a high level of PA-FABP mRNA, while brain, lung, skeletal muscle and pancreas had moderate levels. In liver and kidney tissue levels of PA-FABP transcripts were not detectable.

Discussion

Low-molecular-weight FABPs are abundant (i.e., 1–2% of cytosolic protein) in tissues that are active

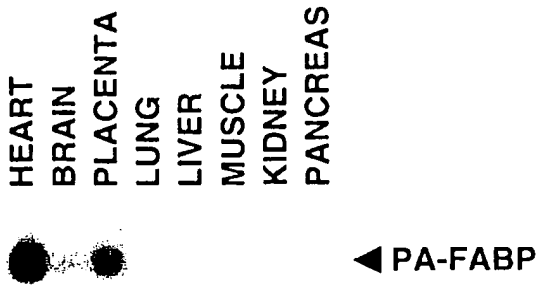

Figure 4. Expression of basal PA-FABP mRNA levels in various adult human tissues.

in fatty acid synthesis, transport, storage, or utilization (15, 16). Whereas the lipid metabolic activity might predict the presence of cytosolic FABPs in epidermis, available data are conflicting. Thus, Schurer et al. (17) found that cytosol from epidermis *in vivo* and *in vitro* showed no fatty acid binding activity within the molecular weight range (12–14 kDa) of these conventional FABPs. However, Siegenthaler et al. (18) characterized an E-FABP (15 kDa) in human epidermal cells that binds oleic acid with high affinity but does not bind all-trans-, 13-cis- and 9-cis-retinoic acid nor all-trans-retinol. They also showed that expression levels of E-FABP were low in normal epidermis, higher in human cultured keratinocytes and still higher in psoriasis. Recently, Madsen et al. (7) also identified a protein (PA-FABP) with homology to other members of the FABP family, that was highly up-regulated in psoriatic skin. Whether PA-FABP and E-FABP are the same or different proteins remains to be investigated.

We have previously cloned and investigated the regulation of a human retinoic acid binding protein (CRABP-II), which is a member of the hydrophobic ligand binding family of proteins that includes FABPs and CRBPs (13, 19, 20). It has been shown that CRABP-II is highly inducible by RA and is also overexpressed in psoriasis (13, 19–21). For this reason we determined whether PA-FABP also was regulated by RA. Interestingly, PA-FABP mRNA was induced following RA treatment for 16 h, whereas no induction was observed, compared to vehicle-treated skin, following SLS treatment for the same period of time. Despite the induction of marked erythema by both SLS and RA after treatment for 96 h, the induction of PA-FABP mRNA by SLS was markedly less than that obtained in response to RA (Fig. 2). It has previously been shown that SLS and RA elicit a similar clinical and histologic response following treatment for 96 h (9). Also, a time-course comparison demonstrated that RA induced epidermal thickening, stratum corneum compaction, spongiosis, and mitotic figures more rapidly than did SLS (22). Taken together, these findings indicate differing mechanisms of actions of the two agents, and that the PA-FABP mRNA induction by RA in human skin *in vivo* cannot be accounted for by irritant effects. A similar effect of RA and SLS on CRABP-II mRNA levels in human skin *in vivo* has also been demonstrated (10).

Glucocorticoids are effective for the treatment of various inflammatory skin diseases including psoriasis. Retinoids exert an opposite effect compared to glucocorticoids and cause hyperplasia of the skin when applied topically (9, 22). It has been shown that retinoids can reverse glucocorticoid-induced atrophy in humans and mice, while the anti-inflammatory property of glucocorticoids is not affected (23, 24). It was therefore of interest to investigate the effects of glucocorticoids on PA-FABP expression. We found that CLO opposed the effect of RA-induced PA-FABP expression in human skin *in vivo* (Fig. 1). Whether this effect of CLO on PA-FABP expression is due to a direct down-regulation of the gene by the glucocorticoid receptor or is a secondary effect remains to be investigated. It was, however, clear from the *in vitro* experiment that PA-FABP transcripts were markedly lower in both unstimulated and stimulated keratinocytes when grown in the presence of hydrocortisone, which favors the possibility that glucocorticoids directly down-regulate the PA-FABP gene (Fig. 3).

Exposure of keratinizing cells to retinoids has been shown to inhibit the normal sequence of keratin expression (25) and cornified envelope formation (26). The extent of keratinocyte differentiation can be enhanced under submerged conditions, when the keratinocytes are cultured in the presence of delipidized serum to reduce the level of RA (25). Furthermore, addition of RA at micromolar concentrations to submerged cultures grown in RA-depleted medium induces marked changes in lipid synthesis and lipid composition with a decreased cholesterol sulfate production (27) and leads to a complete suppression of acylceramide and lanosterol synthesis (28). Our findings that treatment of subconfluent cultures of keratinocytes with RA for up to 48 h showed unaltered or a tendency towards decreased PA-FABP mRNA levels as compared with basic levels might thus possibly reflect an altered lipid metabolism (Fig. 3). In contrast to RA, when grown at physiological calcium concentrations, keratinocytes display a markedly higher capacity to differentiate. Also, both involucrin content and transglutaminase activity, two key determinants of cornified en-

velope during terminal differentiation, are accelerated and begin to occur prior to confluence when the extracellular calcium concentrations are raised to physiological levels (29). PA-FABP mRNA was found to respond to both external calcium (2 mM) (Fig. 3) and FCS (10%) (data not shown) which favors the concept that the stratified structure of the epidermis and/or the presence of dermis is an important determinant of PA-FABP regulation *in vivo*. Taken together, our findings are in accordance to those of Madsen et al. (7) who found that, under conditions that promoted incomplete terminal differentiation (10% FCS), PA-FABP and a few other proteins were strongly up-regulated.

Although the FABPs have functional similarities, their amino acid sequences are unique for each tissue type; but across species the FABP of a specific tissue has a highly conserved amino acid sequence. The tissues containing the most abundant quantities of the FABPs are found in liver (3% of cytosol protein), intestine (1–2% of cytosol protein), and heart muscle (5% of cytosol protein) (30). Using a PAGE-autoradioblotting technique, Siegenthaler et al. (18) demonstrated that liver-, intestine-, and heart- when compared to E-FABP showed distinct mobilities, suggesting that the primary structure of E-FABP may be different in these tissue. The study also showed that E-FABP was detectable in adipose tissues. The present study reveals that PA-FABP mRNA expression is not restricted to epidermal cells since high or moderate message levels were detected in heart, placenta, brain, lung, skeletal muscle and pancreas.

In conclusion, the main findings of the present study show that PA-FABP is regulated by RA in human skin *in vivo* but not in cultured keratinocytes or skin fibroblasts. We also demonstrate that PA-FABP mRNA induction by RA in human skin cannot be explained by its "irritant" properties since PA-FABP mRNA induction after 96 h is markedly less following topical application of the irritant SLS than that obtained in response to topical RA treatment. Although PA-FABP was named such because it is highly up-regulated in psoriatic keratinocytes the present study shows that it is simply an RA-regulated FABP found both in skin and several other tissues.

Acknowledgments

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Structure–function relationships of the liver and muscle isoforms of carnitine palmitoyltransferase I

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Abstract

Elucidation of the membrane topology of carnitine palmitoyltransferase (CPT) I showed that the extreme N-terminus is involved in determining the sensitivity of the liver (L) isoform to malonyl-CoA and suggested that interaction between the two cytosolic segments of the CPT I molecule determines the kinetic characteristics of the enzyme. Work with chimaeric liver/muscle-isoform (L/M) proteins constructed from all six possible combinations of three domains [N-terminus plus transmembrane domain 1 (TM1), loop plus TM2 and C-domain] expressed in *Pichia pastoris* showed that the precise N–C and TM1–TM2 pairings determine the overall kinetic parameters of the protein. Discrete short sequences within the respective N-terminal regions have negative or positive effects on malonyl-CoA sensitivity (L-isoform) or the K_m for carnitine (M-isoform) in the full-length proteins, thus imparting to them their distinctive kinetic characteristics. Interactions within N-terminal domains also seem to be important in the targeting of the protein to microsomes in the *P. pastoris* expression system.

Key words: carnitine palmitoyltransferase, chimaeric proteins, malonyl-CoA, mitochondria, topology.

Abbreviations used: ACBP, acyl-CoA-binding protein; CPT, carnitine palmitoyltransferase; etomoxir, 2-[6-(4-chlorophenoxy) hexyl]oxirane carboxylic acid; L, liver; M, muscle; TM, transmembrane.

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Introduction

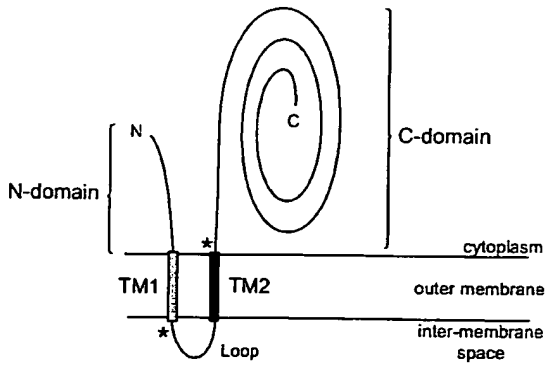
The study of the structure–function relationships of carnitine palmitoyltransferase (CPT) I received three important boosts in the past decade through (1) the cloning and sequencing of the cDNA coding for the liver (L)- and muscle (M)-isoforms [1,2] initially for the rat, and subsequently for other species [3], (2) the demonstration that the mature protein retained intact the N-terminus of

the nascent protein [4], and (3) the elucidation of the membrane topology of the protein in rat liver mitochondria [5]. The second of these observations turned out to be of major importance, in view of the roles that distinct regions of the cytosolic-accessible N-terminal domain are now known to have [6]. To emphasize this point, we start by considering the smallest CPT I entity, or 'catalytic core' [6], that is required to express catalytic activity that is sensitive to both malonyl-CoA and 2-[6-(4-chlorophenoxy)hexyl]oxirane carboxylic acid (etomoxir)-CoA. Such a mutant protein is easily expressed and studied in the yeast *Pichia pastoris*, as are all other mutants discussed in this article [7]. The concept of a catalytic core was developed recently [6] to account for the observation that N-terminally truncated mutants of the L- and M-proteins (Δ1–82 and Δ1–80 respectively) are active and malonyl-CoA-sensitive. These truncations remove the entire N-terminal domain, transmembrane domain 1 (TM1) and part of the inter-TM loop (Figure 1). The most important observation on these truncation mutants is that their respective kinetic characteristics are much more similar to each other than are those of the native L- and M-proteins [6]. Thus the difference in IC_{50} values for malonyl-CoA is narrowed by half, and the K_m for carnitine of the two mutants is very similar (whereas there is a 5-fold difference between the K_m values of the two native proteins, either when expressed in yeast [6] or when measured in isolated rat liver and muscle mitochondria respectively [8]). In contrast, whereas the $K_{0.5}$ for palmitoyl-CoA is very similar for the native proteins, they are significantly different for the two catalytic cores of L- and M-CPT I. It is therefore evident from these results that TM1 plus the N-terminal (cytosolic) 46 residues have a major role in determining the overall kinetics of both L- and M-CPT I.

Figure 1

Membrane topology of CPT I

The asterisks indicate the boundaries of the domains of L- and M-CPT I that were swapped to construct the chimaeric proteins described in the text. See [5] for details of topology.



This was first inferred from work leading up to the elucidation of L-CPT I topology in the mitochondrial outer membrane [5]. It was found that the action of proteinase K on the native enzyme did not decrease the total amount of immunoreactive protein but (1) increased its electrophoretic mobility by the equivalent of approx. 1 kDa and (2) abolished malonyl-CoA sensitivity [5]. Although there was a concomitant loss of catalytic activity (approx. 60%) it preceded that of malonyl-CoA sensitivity [5]. These results, in conjunction with other observations, were interpreted as indicating that the loss of a relatively small number of amino acid residues from the extreme N-terminus of the protein results in the abolition of malonyl-CoA sensitivity [5]. The validity of this conclusion was subsequently confirmed by demonstration that Glu-3 has a central role in positively affecting the malonyl-CoA sensitivity of the L-isoform [9] although not that of the M-isoform (see below).

Chimaeric protein studies

The role of the N-terminal domain is turning out to be much more complex and interesting. We showed, by using six chimaeric proteins constructed from all possible combinations of three (N-terminal plus TM1, TM2 plus loop, and C-terminal) domains/regions of L-and M-CPT I [10], that the precise combinations of the N- and C-terminal domains determine the IC_{50} for malonyl-CoA and the $K_{0.5}$ for palmitoyl-CoA. For M-CPT I, the IC_{50} for malonyl-CoA was not responsive to domain swapping, but the $K_{0.5}$ for palmitoyl-CoA was dependent on the retention of at least one of the native N–C or TM1–TM2 pairings. These results indicated that the C-terminal domain of M-CPT I, unlike that of L-CPT I, can accommodate changes in the N- or C-terminal domain interactions without a loss of affinity for palmitoyl-CoA. In contrast, the K_m for carnitine was found to be highly dependent on the precise TM1–TM2 pairings for both the L- and M-isoforms, but especially for that of M-CPT I. These subtle interactions mean that CPT mutants having a continuous range of values for K_m for carnitine can be constructed by altering N–C and/or TM1–TM2 interactions. The conclusion is that the C-domain alone does not determine the

overall kinetics of the native proteins but that the N-terminal and TM segments exert a major influence. Perhaps the most striking observation was that the kinetics with respect to palmitoyl-CoA and carnitine responded to the swapping of different domains [10].

Deletion mutant studies

However, a question mark remained about the role of the N-domain in determining the sensitivity to malonyl-CoA. Thus, whereas this parameter was sensitive to the disruption of N–C pairing in L-CPT I, such manipulations had no discernible effect on that of M-CPT I [10]. The much higher sensitivity of the latter seemed to be unattainable by L-CPT I through any combination of domain swapping. This paradox was resolved by the observation that the catalytic core of L-CPT I [i.e. the Δ(1–82) L-CPT I mutant] has a malonyl-CoA sensitivity that is much more similar to that of native L-CPT I than to those of either the Δ(3–18) or the E3A mutants. This raised the prospect that the role of the N-terminal domain extends beyond the all-or-none effect of residue Glu-3 in L-CPT I. Certainly, physiologically, a more complex role would be required of any region of the protein involved in modulating its malonyl-CoA sensitivity, as L-CPT I is capable of expressing a wide range of inhibitor sensitivities in response to physiological change [11].

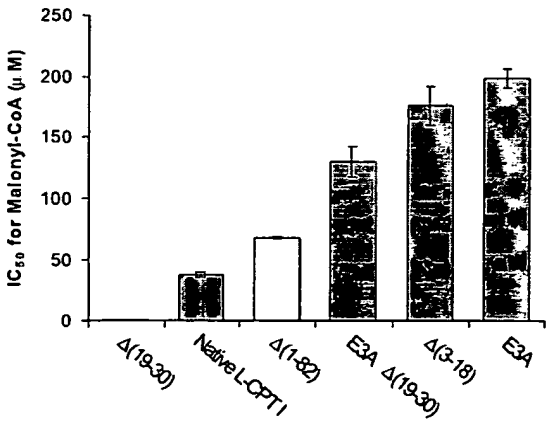
The observation that the Δ(1–82) L-CPT I mutant is severalfold more sensitive than the E3A L-CPT I mutant to malonyl-CoA suggested that a sequence residing between the extreme N-terminus and TM2 acts as a negative determinant of malonyl-CoA sensitivity in L-CPT I. Mutants containing deletions internal to the N-terminal 46-residue cytosolic domain were expressed in *P. pastoris* and their kinetic characteristics were studied. It was found that the Δ(19–30) mutant of L-CPT I had a more than 50-fold higher sensitivity than the native protein to malonyl-CoA, making it even more sensitive than the native M-isoform [6]. This is the strongest evidence yet that the catalytic core of L-CPT I does not determine the malonyl-CoA sensitivity of the native protein but that this parameter can be raised to a level beyond that of native M-CPT I by the removal of a negatively acting sequence (Figure 2). The effect is almost totally dependent on the continued presence of Glu-3 [6]. One possibility is therefore suggested that the sequences that flank either side of the turn predicted to occur around residue Pro-16 might interact to modulate the effect of the cytosolic N-terminal domain on overall malonyl-CoA sensitivity. Recent work (V. N. Jackson, N. T. Price and V. A. Zammit, unpublished work) has indicated that specific residues within this 12-residue stretch of amino acids are responsible for this effect, thus ruling out the possibility that the observed negative effects of this region are due to a molecular spacer action.

Interestingly, the deletion of residues 19–30 from *M*-CPT I did not increase further the already high malonyl-CoA sensitivity of this isoform [6]. This agrees with observations from our and other laboratories that neither a point mutation of Glu-3 nor the truncation of the N-terminal 18 residues have any effect on malonyl-CoA sensitivity, although larger truncations (e.g. Δ2–28) do [6,12]. This contrast between L- and M-CPT I with regard to the function of the predicted N-terminal structure for residues 1–30 might account for the difference in the ability of the two isoforms to respond to physiological change (L-CPT I responds, whereas M-CPT I does not; see [13]). The very close similarity between the primary sequences of this region within L- and M-CPT I suggests that interaction with the rest of the CPT I molecule is central to the mechanism of action. This is indicated by the fact that, although the

Figure 2

Range of malonyl-CoA sensitivities generated by positive and negative elements resident within the N-terminal region of L-CPT I

The sensitivity of the Δ(1–82) mutant (i.e. the catalytic core made up of TM2 and the C-segment of L-CPT I) is intermediate between those of native L-CPT I (in which the N-terminal segment is intact) and mutants lacking Glu-3. The internal deletion Δ(19–30) lowers the IC_{50} by more than 50-fold in comparison with native L-CPT I and Δ(1–82). Reproduced with permission from [6] © The American Society for Biochemistry & Molecular Biology.

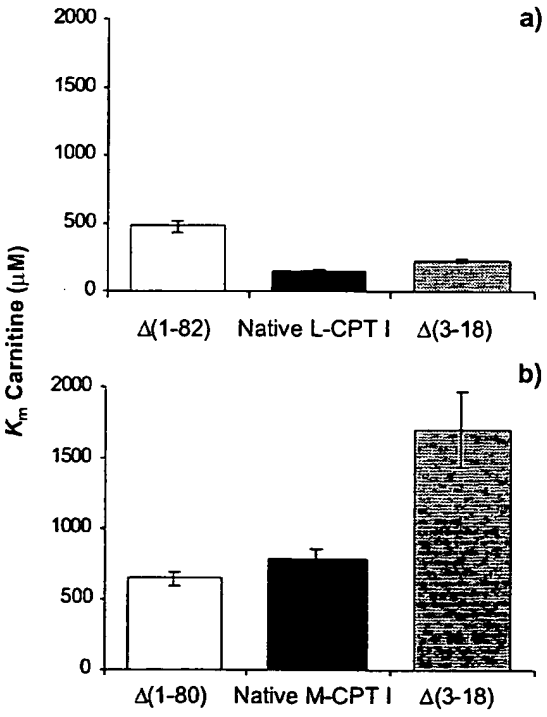


Δ(3–18) and Δ(19–30) mutations do not affect malonyl-CoA sensitivity of M-CPT I, they affect its K_m for carnitine ([6], and V. N. Jackson, N. T. Price and V. A. Zammit, unpublished work). This means that the same motifs that in L-CPT I modify malonyl-CoA sensitivity are involved in determining the affinity of M-CPT I for carnitine (Figure 3). Such a reciprocal action of the respective N-terminal domains might account for the hitherto unexplained inverse relationship between the affinities for malonyl-CoA and carnitine of the two isoforms in isolated rat liver and muscle mitochondria [8]. The near identity of the respective sequences in L- and M-CPT I suggests that it is the mode of their interaction with the malonyl-CoA-binding and carnitine-binding sites on the respective catalytic domains that differs between the two isoforms.

Figure 3

Opposite effects of residues 3–18 on the K_m for carnitine of L- and M-CPT I isoforms

(a) Liver isoform; (b) muscle isoform. Open bars, L-Δ(1–82) and M-Δ(1–80); filled bars, native full-length parental proteins; tinted bars, Δ(3–18) mutants. The K_m values for carnitine of the catalytic cores of L- and M-CPT I are not significantly different. In L-CPT I the presence of the N-segment lowers the K_m for carnitine by 75% in comparison with that of L-Δ(1–82); however, the deletion of residues 3–18 has no effect. The presence of the intact N-terminal region in native M-CPT I does not affect the K_m for carnitine in comparison with that of the M-Δ(1–80) deletion mutant; however, the removal of residues 3–18 increases the K_m 2.5-fold.



Physiological considerations

How do these findings relate to the likely behaviour of the protein in (one of) its natural environment(s): the mitochondrial outer membrane? Until recently, it used to be assumed that the kinetics of the protein was uniform for CPT I in liver mitochondria isolated from animals in any given physiological state. Changes in the malonyl-CoA sensitivity are closely correlated with changes in the membrane's lipid fluidity both *in vivo* [14] and *in vitro* [15], giving rise to the hypothesis that altered TM–membrane and/or TM1–TM2 interactions affect inter-cytosolic domain interactions, and thence the kinetics of the enzyme [16,17]. It has now emerged that CPT I can exist in both the bulk outer membrane and the contact sites of rat liver mitochondria, and indeed that it is enriched in the latter micro-environment [18]. Moreover, the kinetics with respect to long-chain acyl-CoA substrate (palmitoyl-CoA) is markedly different in the two membrane environments. Thus the $K_{0.5}$ for palmitoyl-CoA is 2.5-fold higher for the enzyme in the outer membrane, and the IC_{50} for malonyl-CoA is correspondingly 3-fold higher in the contact sites [19]. Moreover, the inhibition by malonyl-CoA is competitive between malonyl-CoA and palmitoyl-CoA in the contact sites, and non-competitive for the enzyme in the outer membrane (but with the same K_i). These results suggest that CPT I adopts tertiary structures that are sufficiently different in the two membrane environments to alter its palmitoyl-CoA-binding site and/or its interaction with acyl-CoA-binding protein (ACBP), which has been suggested to be the effective substrate of the enzyme [16]. Given the different membrane lipid composition at contact sites and outer membranes, as well as the potential for different protein–protein interactions at the two sites, it is tempting to speculate that the different CPT I kinetics might reflect a change in the intramolecular/intermolecular interactions of the enzyme in response to changes in its membrane environment. Because the number of contact sites is fluid [20] and can be affected by hormones and/or substrates in intact cells [21], it is suggested that the different kinetics of the enzyme populations residing within the two sub-mitochondrial sites could be physiologically important. In particular, the enzyme activity in the outer membrane environment is predicted to be inhibited by malonyl-CoA even at

high palmitoyl-CoA concentrations, in view of the non-competitive nature of the inhibition. Therefore, for this population of CPT I molecules, an increase in fatty acid delivery to the liver (and therefore in ACBP-acyl-CoA substrate) would not by itself be sufficient to overcome the inhibitory effects of malonyl-CoA.

The importance of the N-terminal domain might be even more far-reaching than implied above. We have provided evidence that CPT I might be expressed in microsomes and peroxisomes in rat liver [17,22,23]. The CPT activity and 2,4-dinitrophenyl-etomoxir labelling of an 88 kDa protein are strictly proportional in all of these fractions. Moreover, this protein is immunoreactive (in proportion to its activity) against each of three antibodies raised against linear epitopes from the inter-TM loop (residues 86–100) and the catalytic domain (residues 428–441 and 744–773). Crucially, however, an antibody (anti-N) raised against residues Val-14–Lys-29 of the N-terminal domain was much more reactive with the protein expressed in microsomes than with that in the outer mitochondrial membrane (or peroxisomes) [17,22,23]. Moreover, the differential reactivity with anti-N antibody was also demonstrated (by ELISA) when the protein was in its native state in intact microsomes and mitochondria [22,23]. Interestingly, it has been known for some time that proteolysis of the extreme N-terminus of the protein in intact mitochondria results in the unmasking of this epitope [5]. These results all indicate that CPT I is differentially targeted to microsomes or mitochondria through modification of its N-terminal domain, such that the Val-14–Lys-29 epitope is masked from interaction in the mitochondrial protein but exposed to it in the microsomal protein. Recent findings in this laboratory (F. Fraser, V. N. Jackson, N. T. Price and V. A. Zammit, unpublished work) have shown that specific features of this domain might be involved in such targeting and therefore that the same regions of the N-terminal domain that determine the overall kinetics of the enzyme (see above) might also be involved in determining its targeting to microsomes.

In conclusion, our recent findings show that the overall kinetics of the two isoforms of CPT I are highly dependent on the cytosol-facing N-terminal region of the protein. The observations that this region contains both positive and negative determinants of malonyl-CoA sensitivity in L-CPT I and of affinity for carnitine in M-CPT I suggests that it acts not through direct binding of these ligands but by interacting with the C-domain and/or altering inter-TM-segment interactions. The latter are the most likely molecular entities to sense the changes in membrane lipid properties that are thought to effect the changes in L-CPT I kinetics *in vivo* under different physiological conditions [14]. They might also be involved in mediating the effects of altered membrane microenvironment on CPT I kinetics for the enzyme in the outer membrane and contact sites respectively. The demonstration that the N-terminal domain regions that determine its kinetics are also involved in extra-mitochondrial targeting of the protein raises the exciting prospect of these sequences giving rise to CPT I with different kinetic properties and responses to physiological state [24] in different subcellular locations.

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Further insights into peroxisomal lipid breakdown via α- and β-oxidation

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Abstract

Mammalian peroxisomes degrade fatty carboxylates via two pathways, β-oxidation and, as shown more recently, α-oxidation. The latter process consists of an activation step, followed by a hydroxylation at position 2 and cleavage of the 2-hydroxyacyl-CoA, generating formyl-CoA (precursor of formate/CO_2) and, in case of phytanic acid as substrate, pristanal (precursor of pristanic acid). The stereochemistry of the overall pathway, cofactor requirements and substrate specificity of the hydroxylase and the cleavage enzyme, which is homologous with bacterial oxalyl-CoA decarboxylases, will be discussed. With regard to β-oxidation, peroxisomes contain different acyl-CoA oxidases, multifunctional proteins and thiolases. Based on substrate spectra and stereospecificities of these enzymes, a model was proposed whereby straight chain and branched compounds are degraded by separate pathways. The biochemical findings in mice lacking the D-specific multifunctional protein, however, do not fully support this model. These animals, together with the *Pex5*$^{-/-}$ mice, might be useful to pinpoint the pathological factors contributing to the brain abnormalities in Zellweger patients. Apparently, the deficit in docosahexaenoic acid, presumably formed via peroxisomal β-oxidation, is not the major cause.

Key words: bile acids, cholesterol, peroxin, phytanic acid, pristanic acid, Zellweger syndrome.

Abbreviations used: DHA, docosahexaenoic acid; ER, endoplasmic reticulum; 2HPCL, 2-hydroxyphytanoyl-CoA lyase; MFP, multifunctional protein; PBD, peroxisome biogenesis disorder; Pex, peroxin; PTS, peroxisome targeting signal; RCDP, rhizomelic chondrodysplasia punctata; VLCFA, very-long-chain fatty acid; ZS, Zellweger syndrome.

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Introduction

In mammals, peroxisomes are closely involved in the degradation of isoprenoid-derived lipids either via β-oxidation or, as shown more recently, via α-oxidation. Substrates for the β-oxidation pathways include pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and C_{27} bile acid intermediates [e.g. 3α,12α-dihydroxy-5β-cholestan-26-oic acid ('DHCA') or 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid ('THCA')], both possessing a methyl branch in the α-position of the carboxy group. In addition to these isoprenoids, peroxisomes also degrade straight-chain carboxylates such as very-long-chain fatty acids (VLCFAs), dicarboxylic fatty acids, derivatives of arachidonic acid (prostaglandins and leukotrienes) and certain xenobiotics. It has become clear that the α-oxidative decarboxylation process of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), resulting in the production of pristanic acid, is intimately associated with peroxisomes. Different new enzymes involved in these degradation pathways have been discovered during the past decade and purified, and their cDNA species have been cloned. Analysis of the substrate specificity and stereoselectivity of the β-oxidation enzymes led us to propose the presence of different β-oxidation pathways in peroxisomes (reviewed in [1–3]).

Increased insight into the organization of peroxisomal oxidative metabolism went almost hand in hand with the unravelling of the biogenesis of peroxisomes. Most matrix proteins contain at their C-terminus a tripeptide that acts as a topogenic signal. This peroxisome targeting signal (PTS1), often Ser-Lys-Leu or some variant, is recognized by a cytosolic binding protein, peroxin 5 (Pex5p). A few other peroxisomal matrix proteins contain an N-terminal targeting sequence (PTS2) that interacts with Pex7p [4]. Defects in